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(54) Title: NEUTRALIZING ANTICOAGULANT ACTIVITIES OF LOW MOLECULAR WEIGHT HEPARIN (57) Abstract <p>A system comprising heparinase to degrade polydispersed low molecular weight heparin fractions and fragments (LMHF) both <i>in vitro</i> and <i>in vivo</i>. <i>In vitro</i> data show that both the APTT and anti-FXa activities of LMHF, including Kabi 2165, PK 10169, CY 216 and CY 222, are nearly completely eliminated by heparinase in less than 20 minutes, even when the LMHF are present at a level ten times greater than normally used clinically.</p>		

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NEUTRALIZING ANTICOAGULANT ACTIVITIES
OF LOW MOLECULAR WEIGHT HEPARIN

Description

Background of the Invention

5 This invention is in the general field of
anticoagulant therapy and in particular involves the
degradation and neutralization of heparin.

 Heparin inhibits the proteases of blood coagulation
and is therefore used universally as an effective
10 antithrombotic agent for the treatment and prophylaxis of
many thromboembolic disorders, for maintaining the
fluidity of blood in extracorporeal circulation, and in
surgery of the heart and blood vessels. However, its
clinical use is sometimes associated with hemorrhagic
15 complications. It is assumed that the antithromboplastin
effects of heparin are associated with heparin's anti-FXa
activity, and the hemorrhagic effects are associated with
heparin's potency in prolonging the activated partial
thromboplastin time (APTT). Recently, the dichotomy
20 between the anti-FXa activity and the APTT activity of
heparin has been found to be dependent on the molecular
weight of the heparin, leading to the development of the
low molecular weight heparin fractions and fragments
(LMHF).

25 In numerous experimental models, LMHF have been
shown to have a significantly higher specific anti-FXa
activity than heparin, yet only slightly prolong the
APTT, and therefore have a lesser tendency than heparin
to induce hemorrhage. However, owing to the small
30 molecular size and weight for the LMHF, protamine, a

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conventional antagonist to heparin, can no longer bind with these compounds effectively and neutralize their anticoagulant functions. For certain clinical situations such as kidney dialysis and open heart surgery where a high dose of anticoagulant is required, bleeding risks may be higher with the employment of the LMHF than that of heparin simply because of the lack of an available and appropriate neutralizing agent.

It is therefore an object of the present invention to provide systems for neutralizing and removing low molecular weight heparin fractions and fragments, especially during extracorporeal procedures.

It is further object of the invention to provide a system for removing low molecular weight heparin fractions and fragments which is safe for use both in vitro and in vivo.

It is a still further object of the present invention to provide a system for neutralization of both the APTT and anti-FXa activities of low molecular weight heparin fractions and fragments.

Summary of the Invention

An immobilized heparinase system which can be placed at the termination of an extracorporeal device to neutralize the anticoagulant effects of the high level of heparin encountered during extracorporeal therapy. The heparinase degrades both polydispersed heparin and LMHF, fractions either derived from heparin by fractionation, digestion or degradation, or chemically or biologically synthesized, generally in the range of 2,500 to 6,000 daltons, to inactive, chain-shortened fragments with an average molecular weight of about 1,000 daltons.

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In the given examples, the heparinase system was reacted in vitro with LMHF from different sources, including Kabi 2165, Pharmuca PK 10169, Choay CY 216 and CY 222. Neutralization of both the APTT and anti-FXa
5 anticoagulant activities of these LMHF by heparinase in plasma was also demonstrated.

The immobilized system provides a means for safe, efficacious clinical usage of LMHF in extracorporeal therapy.

10 Brief Description of the Drawings

Figure 1 is a graph of the neutralization of the APTT activity -o-o-o- and anti-FXa activity -o-o-o- of heparin (a) and LMHF: Kabi 2165 (b); PK 10169 (c); CY 216 (d); and CY 222 (e) with protamine.

15 Figure 2 is a graph of the neutralization of the APTT activity -o-o-o- and anti-FXa activity -o-o-o- of heparin (a) and LMHF: Kabi 2165 (b); PK 10169 (c); CY 216 (d); and CY 222 (e) with heparinase.

Detailed Description of the Invention

20 Heparinase is an eliminase which cleaves heparin at α -glycosidic linkages in heparin's major repeating unit: $\rightarrow 4$)-2-deoxy-2-sulfamino- -D-glycopyranose 6-sulfate- (1 \rightarrow 4)- α -L-idopyranosyluronic acid 2-sulfate-(1 \rightarrow . Heparinase (E.C.4.2.2.7) isolated from Flavobacterium
25 heparinum is purified to homogeneity by a combination of hydroxylapatite chromatography, repeated gel filtration chromatography, and chromatofocusing as described by Yang et al. in "Purification and Characterization of Heparinase from Flavobacterium heparinum", J. Biol. Chem.
30 260(3), 1849-1857 (1985). Heparinase can also be

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purified by a combination of hydroxylapatite chromatography and negative adsorption on QAE-Sephadex at pH 8.3, as described in "Purification and Characterization of Catalytically Pure Heparinase" by Yang et al. in Biotechnology Progress (in press). The enzyme is extremely specific, having previously been reported to act only on heparin and heparin monosulfate out of twelve similar polysaccharide substrates. Even the heparin monosulfate is not a good substrate: the extent of reaction is only about 28%.

Heparin is used clinically, both in vitro and in vivo, to inhibit blood coagulation. Heparin, a mucopolysaccharide with a wide range of molecular weights of up to 20,000, average molecular weight 13,500, works by directly inhibiting FXa and other serine esterases in the blood and indirectly by interaction with Antithrombin III to inhibit FXa and other serine esterases. The anticoagulant effect is neutralized clinically by the addition of protamine, low molecular weight proteins which form a stable salt with heparin.

Protamine has long been used in conjunction with heparin therapy as a heparin antagonist. A dosage of 1.3 mg of protamine sulfate generally is used in clinical practice to neutralize 100 USP units of heparin. Protamine binds heparin chemically and displaces it from its binding site on the antithrombin molecules, rendering heparin's anticoagulant effects ineffective. It has been suggested that binding requires a minimum of 16 saccharide units. Since LMHF having a molecular weight between 2,500 and 5,000 daltons, prepared by depolymerization of normal heparin, are composed of 8-16 saccharide units, they display a very weak affinity for

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protamine. Several groups have reported the lack of both in vitro and in vivo effectiveness of protamine in neutralizing the anticoagulant effects of LMHF.

When protamine sulfate is supplemented at a dosage
5 most efficient for neutralization (i.e., the apparent equivalent value), more than 40% of the APTT and 60% of the anti-FXa activities of LMHF remain unneutralized. The lack of interaction with protamine, as well as the absence of a proper antagonist to LMHF, excludes the LMHF
10 from some major potential therapeutic applications, such as in extracorporeal therapy, due to concern regarding the risk of bleeding associated with applications requiring a high dose of anticoagulants.

The present invention is the discovery that,
15 unexpectedly, heparinase reacts with LMHF having molecular weights between 2,500 and 6,000, as well as heparin with an average molecular weight of 13,500, degrading them into fragments of molecular weights of approximately 1000 and neutralizing both the anti-FXa
20 activity and the APTT activity.

Table I summarizes the results of the enzymatic degradation of heparin and LMHF with heparinase. As indicated by the different Km values, heparinase appears to exhibit completely different affinities for different
25 LMHF, despite similar manufacturing procedures and even similar molecular weight distributions. The molecular weights and other properties are shown in Table II for these LMHF. For instance, while both Kabi 2165 and PK 10169 display similar molecular weights, PK 10169 shows a
30 Km value ten times higher than that of Kabi 2165. This difference in Km values may be accounted for in terms of the different molecular compositions and/or chemical

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structures present in these compounds. However, almost equal molar quantities of final degradation products are produced by each gram of heparin and LMHF, with the exception of PK 10169, as well as almost identical average molecular weight of the final degradation products.

TABLE IDegradation of Heparin and LMHF by Heparinase

	Average Molecular Weight	Km (M)	Moles of Degrada- tion Products/per gram of substrate	Average Molecular Weight of Degrada- tion Products
10 Heparin	13,500	0.09	0.94	1,060
Kabi 2165	5,000	0.15	0.96	1,040
PK 10169	4,500	1.00	0.81	1,410
15 CY 216	4,500	0.60	0.90	1,110
CY 222	2,500	0.90	0.92	1,090

The following materials and methods were used to obtain the data shown in Table I and II:

Heparin, sodium salt from porcine intestinal mucosa, was purchased from Hepar (Franklin, Ohio). LMHF were obtained from several sources. Kabi 2165 was a gift from Dr. Holmer and Dr. Anderson at KaviVitrum AB, Stockholm. PK 10169 was generously supplied by Dr. Mardigian at the

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Pharmuca Laboratories, Gennevilliers, France. CY 216 and CY 222 were kindly provided by Dr. Choay at the Institute of Choay, Paris, France. The mean molecular weights, specific activities in anti-FXa units and APTT units per mg and relative ratios of anti-FXa activity over APTT activity for heparin and each of the LMHF are listed in Table II.

TABLE IIProperties of Heparin and LMHF

		Mean			Anti-FXa:
	<u>Supplier</u>	<u>Molecular</u>	<u>Anti-FXa</u>	<u>APTT</u>	<u>APTT</u>
		<u>Weight</u>	<u>Units/mg</u>	<u>U/mg</u>	<u>Ratio</u>
10	Heparin	Hepar	13,500	150	1.0
	Kabi 2165	Kabi	5,500	155	3.9
15	PK 10169	Pharmuca	4,500	130	3.1
	CY 216	Choay	4,500	200	6.9
	CY 222	Choay	2,500	250	22.7

Actin Activated Chephaloplastin Reagent (for APTT measurements) was from Dade Diagnostics, Inc., Aguada, Puerto Rico. Substrate S-2222, antithrombin III, Factor Xa, antithrombin III, human normal plasma and buffer (0.05 M Tris, 7.5 mM EDTA, pH 8.4) for the anti-FXa measurements were obtained as a Coatest Heparin Kit from

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Kabi Vitrum, Stockholm. Protamine sulfate (grade III from Herring) was from Sigma Chemical Company, St. Louis, Missouri. Bio-Gel A agarose (8%) was obtained from Bio-Rad Laboratories, Richmond, California. Human blood
5 was collected in citrate (9:1, v/v whole blood to 3.8% weight trisodium citrate) from paid donors at Children's Hospital Medical Center Blood Bank, Boston, Massachusetts. All chemicals were reagent grade, and water was twice distilled.

10

METHODS

Heparinase Preparation and Immobilization

Heparinase, produced by Flavobacterium heparinum, as described by P.M. Galliher et al. in Appl. Environ. Microbiol. 41, 360-365 (1981), was purified using batch
15 hydroxylapatite chromatography, as described by V.C. Yang et al., J. Biol. Chem. 260, 1849-1857 (1985). The partially purified heparinase was used directly or was immobilized on cross-linked 8% agarose beads pre-activated with cyanogen bromide, according to the
20 method described by Bernstein et al. in "An immobilized heparinase system for blood deheparinization", Methods Enzymol. (1986). One unit of heparinase activity was defined as the amount of enzyme which degrades 1 mg of heparin/h.

25 Assay for Activated Partial Thrombin Time (APTT)

100 microliters of Activated Cephaloplastin Reagent prewarmed to 37°C was incubated with 100 microliters of plasma sample at 37°C for 2 minutes, followed by the addition of 100 microliters of 0.02 M CaCl₂. After
30 another 25 seconds, a platinum innoculating loop was drawn through the mixture until a clot was formed and the

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time was recorded. The APTT was compared to a standard curve prepared for each of the individual LMHF at concentrations of 0-0.6 units/ml. The APTT units were defined using the third Heparin International Standard as
5 reference. Samples for measurements were first diluted with normal human plasma until the APTT lay within the linear range of the standard curve.

Assay for Anti-FXa activity

800 microliters of 0.2 M Tris-EDTA buffer (pH 7.4)
10 was mixed with 100 microliters antithrombin III, followed by the addition of 100 microliters of plasma sample. 200 microliters of this mixture was incubated at 37°C and 100 microliters of Factor Xa solution (7 nkat/ml) was added. After 30 seconds, 200 microliters of S-2222 (0.75 mg/ml)
15 was added. The reaction was quenched after 180 seconds by the addition of 300 microliters of 50% acetic acid. The absorbance at 405 nm was measured and compared to a standard curve prepared for each of the individual LMHF at concentrations of 0-0.1 units/ml. The anti-FXa units
20 were defined using the 3rd Heparin International Standard in the Yin and Wessler test, J. Lab. Clin. Med. 81, 298-310 (1973). Samples for measurements were first diluted with human plasma until their absorbance at 405 nm lay within the linear range of the standard curve.

25 Degradation of LMHF by Heparinase in Aqueous Buffer

Heparinase is an eliminase which cleaves heparin at the 1,4 glycosidic linkage to produce α , β unsaturated uronides. The non-reducing end of the chain-shortened heparin fragments is a chromophore which possesses an
30 absorption maximum at 232 nm. The degradation of LMHF by

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- heparinase is monitored by the appearance of these ultraviolet adsorbing heparin fragments. One ml of each of the LMHF preparations (25 mg/ml) is mixed with 0.1 ml of heparinase solution (0.3 mg/ml, specific activity: 5 200 units per mg of protein) and incubated at 37°C. At various times, 25 microliters of the reaction mixture is withdrawn and quenched in 1.5 ml of 0.03 M HCl. The time-dependent increase in absorbance at 232 nm is used to calculate the initial rate of the reaction. To 10 determine the Michaelis-Menten rate constant (K_m) of the heparinase reaction for each of the LMHF, the initial rates of degradation are measured as a function of the substrate concentration ranging from 0.1 - 25 mg/ml. The K_m value is then estimated from the slope of the 15 Lineweaver-Burke plot. The molar quantities of the degradation products, generated by each milligram of the LMHF sample, as well as the average molecular weight of the degradation products, is estimated from the absorbance at 232 nm in the digestion media after 20 degradation is complete, using an extinction coefficient of $5.1 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ for the α , β unsaturated uronides, as reported in J. Biol. Chem. 245, 6170-6175 (1970). Unless otherwise stated, the buffer contains 0.25 M sodium acetate, 2.5 mM calcium acetate at pH 7.
- 25 Neutralization of LMHF by Protamine in Human Plasma
- Normal heparin (from Hepar) is prepared at a concentration of 0.01 mg/ml in human plasma, while LMHF are prepared at a concentration of 0.1 mg/ml. To nine parts of the heparinized plasma one part of the protamine 30 solution at concentrations ranging from 0.05 to 3.0 mg/ml is added. After 5 minutes of incubation at 37°C the

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residual APTT and anti-FXa activities are measured. The residual activity is represented as the relative activity assuming a 100% value for the initial activity.

Neutralization of LMHF by Immobilized Heparinase

5 Plasma containing heparin or LMHF is prepared as described above. To 9 ml of the heparinized plasma, 5 ml of cross-linked 8% beads (a 75% suspension in physiologic saline solution) containing immobilized heparinase (145 units/ml of beads) is added. The mixture is incubated at
10 37°C with gentle agitation. At time intervals of 0, 10, 20, 40, and 60 minutes during the incubation, an aliquot of 1 ml of the mixture is withdrawn and centrifuged at 3,000 g, 0°C, for 3 minutes to remove the beads and quench the reaction. The residual APTT and anti-FXa
15 activities in the supernatant are measured. The residual activity is represented as the relative activity assuming a 100% value for the initial activity.

Figure 1 shows the neutralization of heparin and LMHF by protamine. When protamine is supplemented at
20 0.03 mg/ml, a complete neutralization of both APTT and Anti-FXa activities is observed for heparin. This value is in good agreement with the figure that is widely accepted and commonly used in clinical practice, 1-2 mg of protamine for each 100 USP units of heparin. The
25 prolongation in APTT and the increase in anti-FXa activity corresponding to the further addition of protamine beyond the equivalent value is a common phenomenon. It is believed to be related to protamine's own anticoagulant properties.

30 Neutralization of LMHF by protamine follows a similar pattern to that of heparin. Both APTT and

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anti-FXa activities decrease with the increase in protamine concentration until they reach a minimum, at which the protamine concentration is defined as the apparent equivalent value and then increase with further increases in protamine concentration. However, neither of these activities are ever completely neutralized by protamine for any of the LMHF samples tested. Even at the apparent equivalent values, at least 40% of the APTT activity still remains in plasma (Figure 1). The anti-FXa activity exhibits a stronger resistance to protamine neutralization than that of the APTT activity, and is insensitive to neutralization over a broad range of protamine concentrations. The degree of neutralization, as well as the apparent equivalent value, varies with the sample used for testing. These results support the findings reported by others that protamine is not an efficient antagonist to LMHF.

Figure 2 shows the neutralization of heparin and LMHF by heparinase. In all cases, including that with normal heparin, more than 80% of both APTT and anti-FXa activities are abolished in less than 20 minutes. After 20 minutes, a nearly complete neutralization of both activities is noted. The residual activities drop to a level of less than 7% of their initial values, and remain unchanged thereafter. Since heparinase is present in a large excess over the level required for complete neutralization, the kinetics of neutralization and the rate of neutralization is almost identical for each of the cases despite the different K_m values for these LMHF. The small amount of APTT and anti-FXa activities (less than approximately 7%) remaining in each of the samples

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after the heparinase neutralization is believed to be attributed to degradation products.

One approach to eliminating bleeding problems associated with extracorporeal heparin therapy consists of placing a blood filter containing immobilized heparinase at the effluent of the extracorporeal device. The filter converts heparin residing in the extracorporeal device to small saccharides with minimal anticoagulant activity before the blood returns to the patient. Data show that such a filter is capable of removing up to 90% of heparin in a single pass for in vitro and in vivo uses.

LMHF have advantages as new antithrombotic agents or heparin substitutes since they present a lower risk of bleeding than heparin, a reduced tendency to cause thrombocytopenia and exhibit a longer in vivo half-life than heparin. Heparinase can now be bound to a blood filter to remove LMHF from the blood. In vitro data demonstrates that LMHF are degraded by heparinase in buffer in a fashion similar to that of heparin but that the kinetics of degradation are somewhat different, presumably because of differences in the chemical nature of the compounds. In plasma, both the APTT and anti-FXa activities of the LMHF are reduced by heparinase to less than 7% of their initial values within a period of 20 minutes. The time required for a complete neutralization can be shortened considerably by employing more enzyme in the system. Trace amounts of anticoagulant activities remaining in the system after heparinase neutralization is not unanticipated. Heparinase degrades heparin to produce products which have a distribution of approximately 50, 30, 15, and 5% for di-, tetra-, hexa-,

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and oligo-saccharide molecules. The oligosaccharides have been reported to possess both APTT and anti-FXa activities. The remaining activities are therefore believed to be attributed to the oligosaccharides.

5 The heparinase system presented here appears to be thus far the only means to effectively neutralize the anticoagulant activities of LMHF. With some modification it also can be used for in vitro quantitation, and even for the in vitro titration of LMHF. In addition, the
10 heparinase system may be of use in furthering clinical acceptance of LMHF in extracorporeal therapy, as well as in their development as improved and "all around" antithrombotic agents.

15 The present invention of using heparinase to degrade low molecular weight heparin fractions and fragments may be embodied in other specific forms without departing from the spirit and scope thereof. Such other embodiments and modifications are intended to fall within the scope of the appended claims.

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CLAIMS

1. A system for neutralizing low molecular weight heparin fractions and fragments comprising heparinase.
- 5 2. The system of Claim 1 wherein said heparinase is immobilized on a solid matrix.
3. The system of Claim 2 wherein said heparinase is immobilized on a solid matrix at a specific level of enzymatic activity per unit of surface area.
- 10 4. The system of Claim 1 wherein said heparinase is purified by a combination of hydroxyapatite chromatography and QAE-Sephadex adsorption.
5. The system of Claim 2 wherein said heparinase is immobilized on inert spherical matrices and
15 contained within a reaction chamber.
6. The system of Claim 5 wherein said chamber is connected to an extracorporeal circuit.
7. The system of Claim 1 wherein said heparinase is calibrated against a known standard for use in
20 quantitating the quantity of heparin in a solution.
8. The system of Claim 7 wherein said solution is blood.

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9. The system of Claim 1 wherein said heparinase is Flavobacterial heparinase.

FIGURE 1A

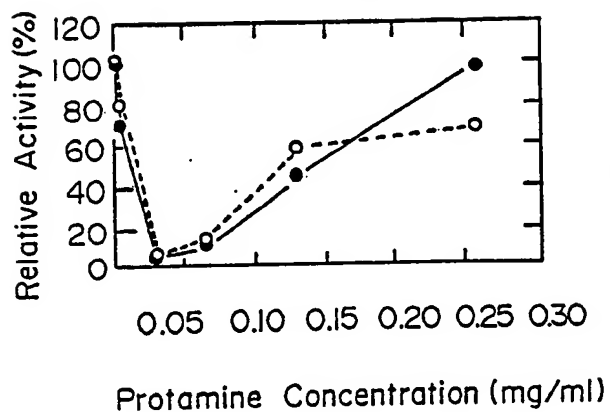


FIGURE 1D

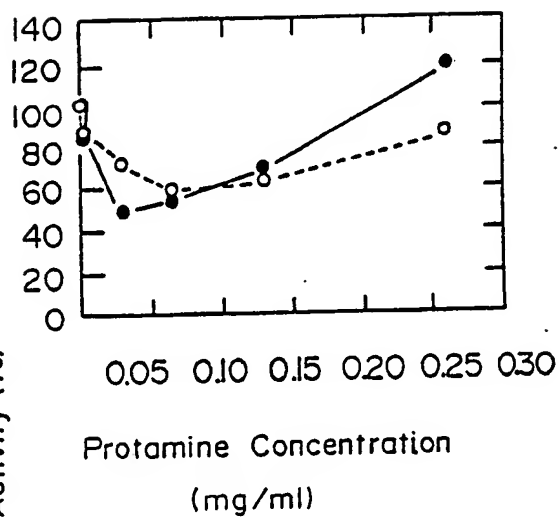
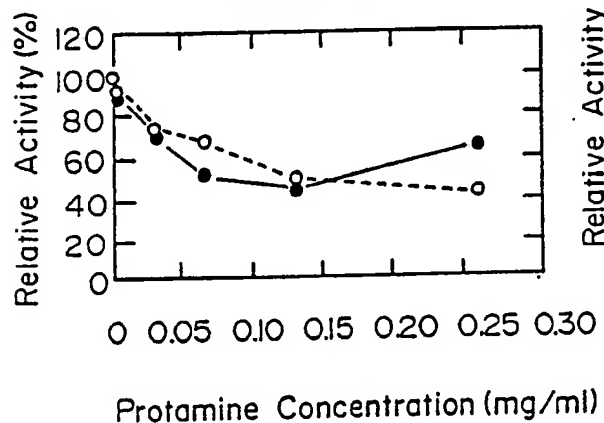


FIGURE 1B



Relative Activity (%)

FIGURE 1E

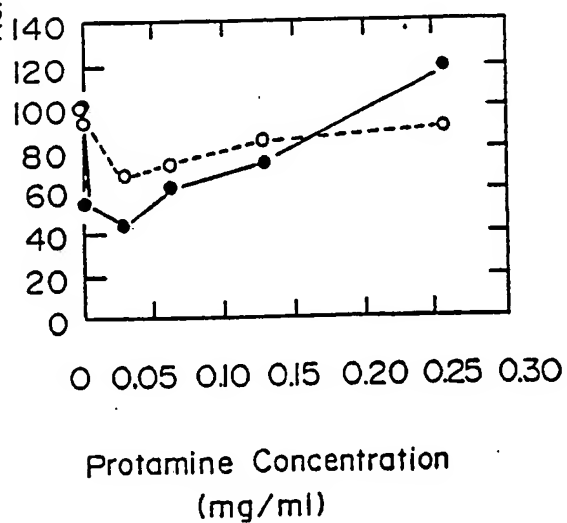


FIGURE 1C

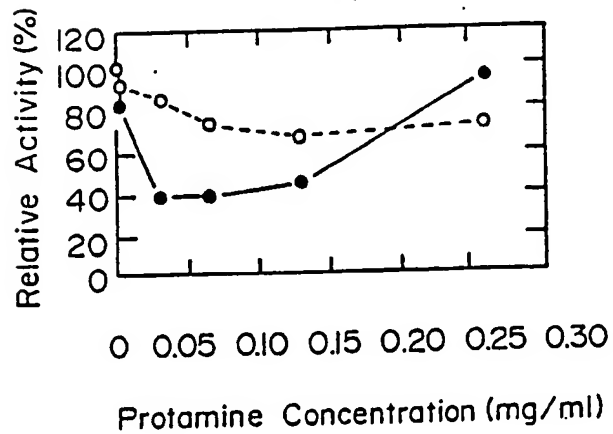
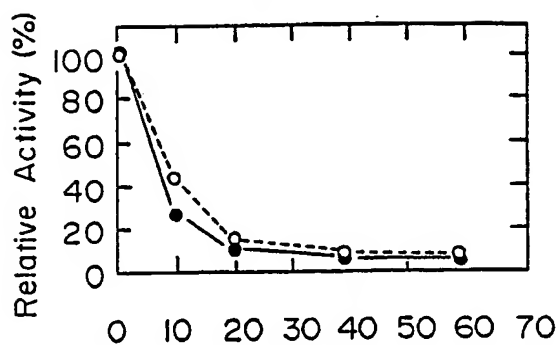
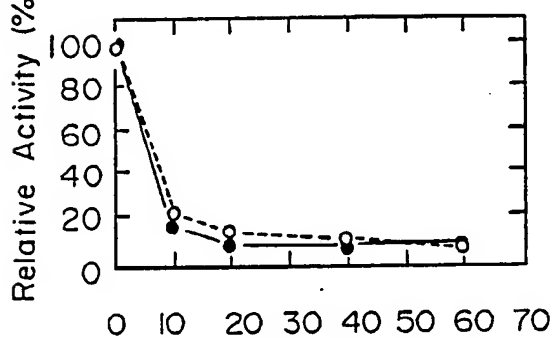


FIGURE 2A



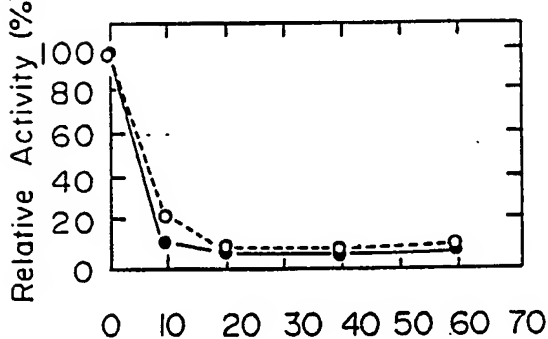
Time (minutes)

FIGURE 2B



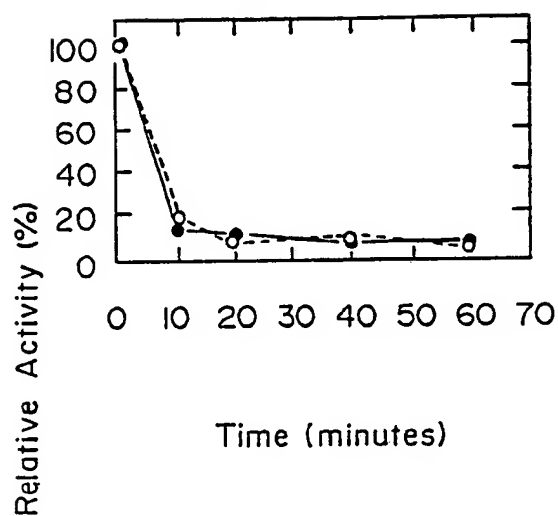
Time (minutes)

FIGURE 2C



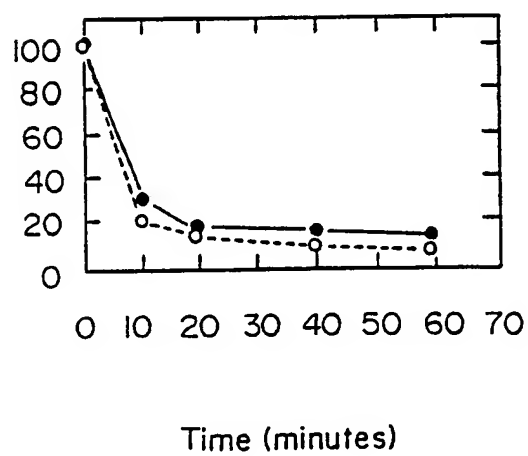
Time (minutes)

FIGURE 2D



Time (minutes)

FIGURE 2E



Time (minutes)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 87/02510

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 12 N 11/10; A 61 M 1/36; C 12 Q 1/24		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 12 N; A 61 M	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US, A, 4373023 (LANGER et al.) 8 February 1983 see the whole document	1-9
Y	--	4
Y	Chemical Abstracts, vol. 102, no. 13, 1 April 1985 (Columbus, Ohio, US) V.C. Yang et al.: "Purification and characterization of heparinase from Flavobacterium heparinum", see page 297, abstract no. 108647e, & J. Biol. Chem. 1985, 260(3), 1849-57 cited in the application	4
X	Chemical Abstracts, vol. 101, no. 4, 23 July 1984 (Columbus, Ohio, US) R.J. Linhardt et al.: "An immobilized microbial heparinase for blood dehepa- rization", see page 290, abstract no. 28237k & Appl. Biochem. Biotechnol. 1984, 9(1) 41-55	1-9 ./.
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
18th January 1988		25 FEB 1988
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC NO SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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X	Chemical Abstracts, vol. 101, no. 22, 26 November 1984 (Columbus, Ohio, US) H. Bernstein et al.: "Design of an immobilized enzyme reactor for removing heparin at the termination of extracorporeal therapy", see page 372, abstract no. 198106j & Polym. Mater. Sci. Eng. 1984, 51, 204-7 -----	1-9
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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 8702510

SA 19150

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/02/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4373023	08-02-83	None	